

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁴ : A23C 19/11, A61K 37/02		A1	(11) International Publication Number: WO 89/12399 (43) International Publication Date: 28 December 1989 (28.12.89)
 (21) International Application Number: PCT/US89/02625 (22) International Filing Date: 16 June 1989 (16.06.89)		 (74) Agents: RYAN, M., Andrea et al.; White & Case, 1155 Avenue of the Americas, New York, NY 10036 (US).	
 (30) Priority data: 209,861 22 June 1988 (22.06.88) 317,626 1 March 1989 (01.03.89)		US	 (81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), HU, IT (European patent), JP, KR, LU (European patent), MC, NL (European patent), NO, SE (European patent), SU.
 (71) Applicant: PUBLIC HEALTH RESEARCH INSTITUTE OF THE CITY OF NEW YORK [US/US]; 455 First Avenue, New York, NY 10016 (US).		 Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
 (72) Inventors: BLACKBURN, Peter ; 426 W. 44th Street, New York, NY 10036 (US). POLAK, June ; 57 Montague Street, Brooklyn, NY 11201 (US). GUSIK, Sara-Ann ; 317 First Avenue, New York, NY 10003 (US). RUBINO, Stephen, D. ; 111 Henry Avenue, Harrison, NY 10528 (US).			

(54) Title: Nisin compositions for use as enhanced, broad range bactericides

(57) Abstract

Bacteriocin compositions comprising lanthionine containing bacteriocins and non-bactericidal agents. When the bacteriocin compositions are combined with a suitable carrier with each component present in sufficient quantities such that the composition is effective against Gram negative bacteria in addition to Gram positive bacteria, they become enhanced, rapid acting, broad range bactericides suitable for a variety of applications.

DescriptionNisin Compositions For Use As
Enhanced, Broad Range BactericidesBackground of the Invention

This is a continuation-in-part application of Serial No. 209,861 filed June 22, 1988. Nisin is a polypeptide with antimicrobial properties which is produced in nature by various strains of the bacterium Streptococcus lactis. It is a known food preservative which inhibits the outgrowth of spores of certain species of Gram positive Bacilli.

Although sometimes mistakenly and imprecisely referred to as an antibiotic, nisin is more correctly classified as a bacteriocin, i.e. a proteinaceous substance produced by bacteria and which has antibacterial activity only towards species closely related to the species of its origin. Nisin is a naturally-occurring preservative found in low concentration in milk and cheese, and is believed to be completely non-toxic and non-allergenic to humans.

Nisin has recently been recognized as safe by the FDA as a direct food ingredient in pasteurized cheese spread, pasteurized processed cheese spread, and pasteurized or pasteurized processed cheese spread with fruits, vegetables, or meats. Furthermore, since it is a polypeptide, any nisin residues remaining in foods are quickly digested.

A summary of nisin's properties appears in Hurst, A., Advances in Applied Microbiology 27:85-123 (1981). This publication describes what is generally known about nisin. Nisin, produced by Streptococcus lactis, is available commercially as an impure preparation, Nisaplin™,

from Aplin & Barrett Ltd., Dorset, England and can be obtained by isolating naturally- occurring nisin from cultures of Streptococcus lactis and then concentrating the nisin according to known methods. There are also reported methods for producing nisin using altered strains of Streptococcus. See Gonzalez et al., U.S. Pat. No. 4,716,115, issued December 29, 1987. It should also be possible to produce nisin by recombinant DNA technology.

Nisin has been applied effectively as a preservative in dairy products, such as processed cheese, cream and milk. The use of nisin in processed cheese products has been the subject of recent patents. See U.S. Pat. Nos. 4,584,199 and 4,597,972. The use of nisin to inhibit the growth of certain Gram positive bacteria has been well documented. However, its complete success and acceptance as a food preservative has heretofore been hampered by the belief that nisin was ineffective against Gram negative and many Gram positive bacteria. Gram negative bacteria are almost always present in conjunction with Gram positive bacteria and are a major source of food spoilage and contamination. See Taylor, U.S. Pat. No. 5,584,199, issued April 22, 1986 and Taylor, U.S. Pat. No. 4,597,972, issued July 1, 1986; Tsai and Sandine, "Conjugal Transfer of Nisin Plasmid Genes from Streptococcus Lactis 7962 to Leuconostoc Dextranicum 181, Applied and Environmental Microbiology, Feb. 1987, p. 352; "A Natural Preservative," Food Engineering Int'l, May 1987, pp. 37-38; "Focus on Nisin," Food Manufacture, March 1987, p. 63.

Summary of the Invention

It has now been found that contrary to prior teaching, compositions comprising nisin, in combination with various non-bactericidal agents have enhanced, broad range bactericidal activity against Gram negative bacteria as well as enhanced activity against a broader range of Gram posi-

tive bacteria than nisin alone. The enhanced bactericidal activity against Gram positive bacteria occurs in a pH range broader than previously taught. The invention provides bacteriocin compositions of nisin or other, lanthionine containing bacteriocins, in combination with various non-bactericidal agents for example chelating agents or surfactants. The invention further provides the compositions dissolved or suspended in a suitable carrier to yield enhanced broad range bactericides.

Detailed Description of the Invention

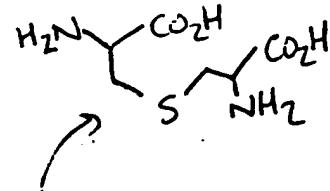
Specifically, it has been found that a solution of about $0.1\mu\text{g}/\text{ml}$ to $300\mu\text{g}/\text{ml}$ of nisin in the presence of about 0.1 mM to 20 mM of a chelating agent, for example EDTA, virtually eliminates the growth of Gram negative bacteria such as Salmonella typhimurium, Escherichia coli, Pseudomonas aeruginosa, Bacterioides gingivalis, Actinobacillus actinomycetemcomitans, and Klebsiella pneumoniae and is more active towards Gram positive bacteria such as Staphylococcus aureus, Streptococcus mutans, Listeria monocytogenes Streptococcus agalactiae and Coryneform bacteria than nisin alone. Although the enhancement of nisin activity by chelator was concentration dependent, contrary to expectations, concentrations of EDTA in excess of 20mM were inhibitory to the bactericidal activity of nisin. However, in the presence of a proteinaceous carrier, and polyvalent polymers such as serum albumin, collagen, gelatin, casein and keratin, the inhibition of nisin by concentrations of EDTA above 20mM was significantly reduced, thereby extending the useful range of EDTA enhancement of nisin.

It has also been found that a solution of about $0.1\mu\text{g}/\text{ml}$ to $300\mu\text{g}/\text{ml}$ nisin and about 0.1 mM to 20 mM of a chelating agent will further enhance the effectiveness of nisin against Gram negative and Gram positive bacteria in

the presence of about 0.01% to 1.0% of surfactant. Additionally, it has been found that, in the presence of surfactant alone, nisin has enhanced activity against Gram positive bacteria.

In the present invention, suitable chelating agents include, but are not limited to, EDTA, CaEDTA, CaNa₂EDTA, and other alkyl diamine tetraacetates, EGTA and citrate. Surfactants, valuable as cleansing agents, suitable for combination with nisin, with or without EDTA, include, but are not limited to, the nonionic surfactants Tweens, Tritons, and glycerides, ionic surfactants such as fatty acids, quaternary compounds, anionic surfactants such as sodium dodecyl sulphate and amphoteric surfactants such as cocamidopropyl betaine and emulsifiers.

Since Gram positive and Gram negative bacteria are almost always found together in foods, the effectiveness of the nisin compositions towards Gram negative bacteria such as Salmonella typhimurium, Escherichia Coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Bacterioides gingivalis, Actinobacillus actinomycetemcomitans, and other Gram negative pathogens and Gram positive bacteria will be of great use. The bactericides are particularly suited for the control and prevention of contamination of raw ingredients, processed foods and beverages by bacterial pathogens and other microbial spoilage organisms. Potential food related uses include treatment of meats, especially poultry, eggs, cheese and fish and treatment of food packaging and handling equipment. Further uses include as food preservative, such as in processed cheese, cream, milk, dairy products and in cleaning poultry, fish, meats, vegetables, and dairy and food processing equipment. The use of the nisin compositions should not be limited to food related uses and the nisin compositions should be useful in any situation in which there is a need or desire to eliminate Gram negative and Gram positive bacteria.

Claims

1. A composition comprising a lanthionine containing bacteriocin and a chelating agent.
2. A composition comprising a lanthionine containing bacteriocin and a surfactant.
3. A composition comprising a lanthionine containing bacteriocin, a chelating agent and a surfactant.
4. The composition as defined in claim 1, 2 or 3 wherein the lanthionine containing bacteriocin is selected from the group consisting of nisin, subtilin, epidermin, cinnamycin, duramycin, ancovenin and Pep 5.
5. The composition as defined in claim 1 or 3 wherein the chelating agent is selected from the group consisting of alkyldiamine tetraacetates, CaEDTA, Na₂CaEDTA, EGTA and citrate.
6. The composition as defined in claim 5 wherein the alkyldiamine tetraacetate is EDTA and the bacteriocin is nisin.
7. The composition as defined in claim 2 or 3 wherein the surfactant is selected from the group consisting of Tritons, Tweens, glycerides, fatty acids, emulsifiers, quaternary compounds, amphoteric and anionic surfactants.

8. The composition as defined in claim 1 also containing a food preservative.
9. An enhanced broad range bactericide comprising a carrier, a lanthionine containing bacteriocin and a chelating agent.
10. An enhanced broad range bactericide comprising a carrier and a lanthionine containing bacteriocin and a surfactant.
11. An enhanced broad range bactericide comprising a carrier, a lanthionine containing bacteriocin, a chelating agent and a surfactant.
12. The enhanced broad range bactericide as in claim 9, 10 or 11 wherein the lanthionine containing bacteriocin selected from the group consisting of nisin, subtilin, epidermin, cinnamycin, duramycin, ancovenin and Pep 5 and the chelating agent selected from the group consisting of alkyldiamine tetraacetates, EGTA and citrate are present in quantities such that the bactericide has enhanced effectiveness against at least one of the bacteria from the group consisting of Staphylococcus aureus, Streptococcus mutans, Listeria monocytogenes, Streptococcus agalactiae, Coryneform bacteria, Salmonella typhimurium, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Bacterioides gingivalis and Actinobacillus actinomycetemcomitans.

13. The enhanced broad range bactericide as in claim 12 wherein the alkyldiamine tetraacetate is EDTA.
14. The enhanced broad range bactericide as in claim 10 or 11 wherein the surfactant is selected from the group consisting of Tritons, Tweens, glycerides, fatty acids, quaternary compounds, emulsifiers, amphoteric and anionic surfactants and is present in an amount sufficient such that the bactericide has enhanced effectiveness against at least one of the bacteria from the group consisting of Gram negative and Gram positive bacteria.
15. The enhanced broad range bactericide as in claim 12 wherein the concentration of nisin is between about 0.1 μ g/ml and 300.0 μ g/ml and the concentration of chelating agent is between about 0.1 mM and 20mM.
16. The enhanced broad range bactericide as in claim 14 wherein the concentration of surfactant is between about 0.01% and 1.0%.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/02625

I. CLASSIFICATION F SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC⁴: A 23 C 19/11, A 61 K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
IPC ⁴	A 23C, A 61 K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	GB, A, 738655 (APLIN & BARRETT LTD) 19 October 1955, see page 2, line 115 - page 3, line 28 --	1-16
A	Chemical Abstracts, vol. 86, no. 1, 3 January 1977, (Columbus, Ohio, US), A.I. Pedenko et al.: "Effect of the antibiotic nisin on pathogenic staphylococci and streptococci" see page 58, abstract no. 594x & Tr. S'ezda Mikrobiol. Ukr. 4th 1975, 221-2	1-16

- Special categories of cited documents: ¹⁰
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "B" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "Z" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

2nd October 1989

Date of Mailing of this International Search Report

24 OCT 1989

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

T.K. WILLIS